Supplementary Information for:

Reactivity-Based Probe of the Iron(II)-Dependent Interactome Identifies New Cellular Modulators of Ferroptosis

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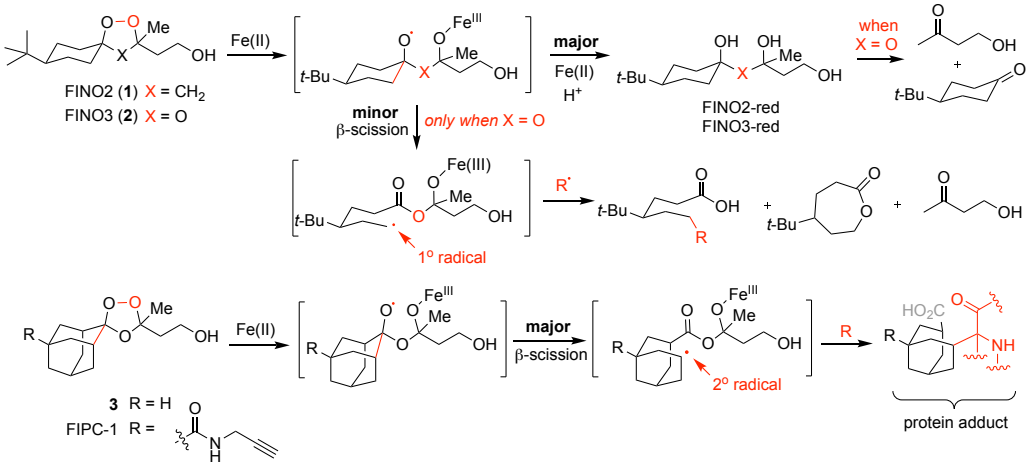
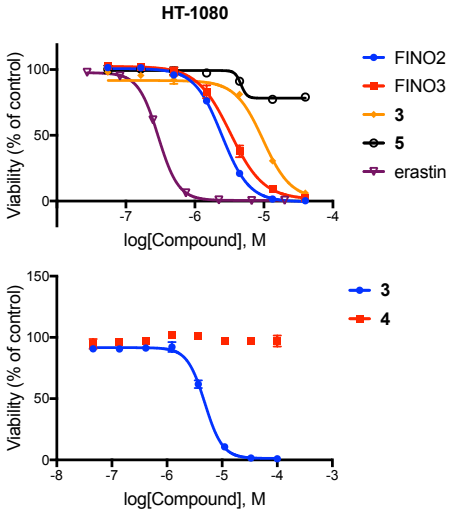


Figure S1. Predicted ferrous iron reactivity of compounds **1**–**3** and FIPC-1 based on previous studies of antimalarial 1,2,4-trioxolanes and 1,2-dioxolanes.[1] Dioxolanes favor a second reduction step over β-scission, while the latter process is disfavored in trioxolane **2** because it would produce a primary carbon-centered radical (top).



compound	EC_{50} (μM)
FINO2 (**1**)	2.5
FINO3 (**2**)	3.2
3	9.7
4	>>100
5	>100

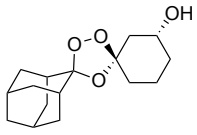


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Figure S2. Cytotoxicity of FINO2, FINO3 (**2**), and compounds **3**–**5** in HT-1080 cells. Representative dose response curves with the table summarized with EC_{50} numbers.

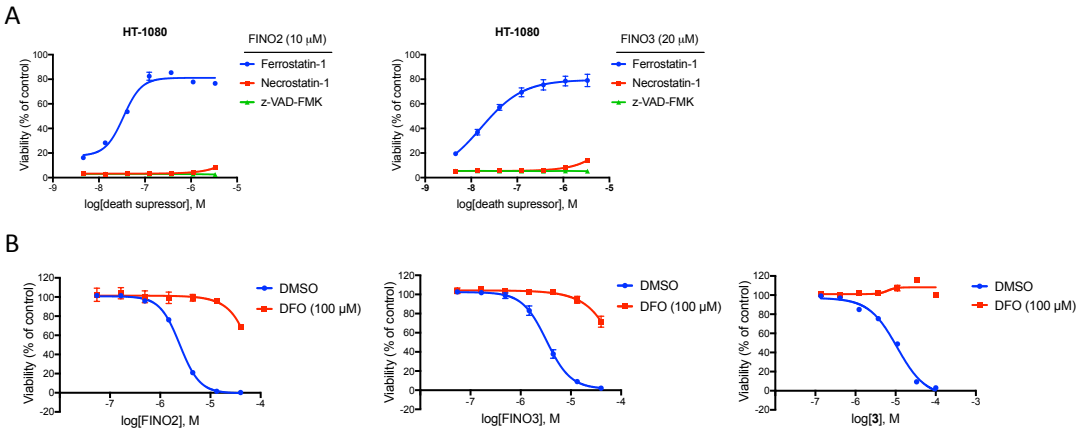


Figure S3. FINO3 and **3** induce ferroptosis in HT-1080 cells. A. Cell death induced by FINO2 (**1**) and FINO3 (**2**) is effectively rescued by ferrostatin-1 but not necrostatin-1 or z-VAD-FMK. HT-1080 cells were co-treated with FINO2 (10 μM) or FINO3 (20 μM) and a dilution series of a protection molecule. Cell viability was measured after 24 h incubation. B. DFO (100 μM) rescues cell death induced by FINO2, FINO3 (**2**), or **3**. Cell viability was measured after 24 h of co-incubation.

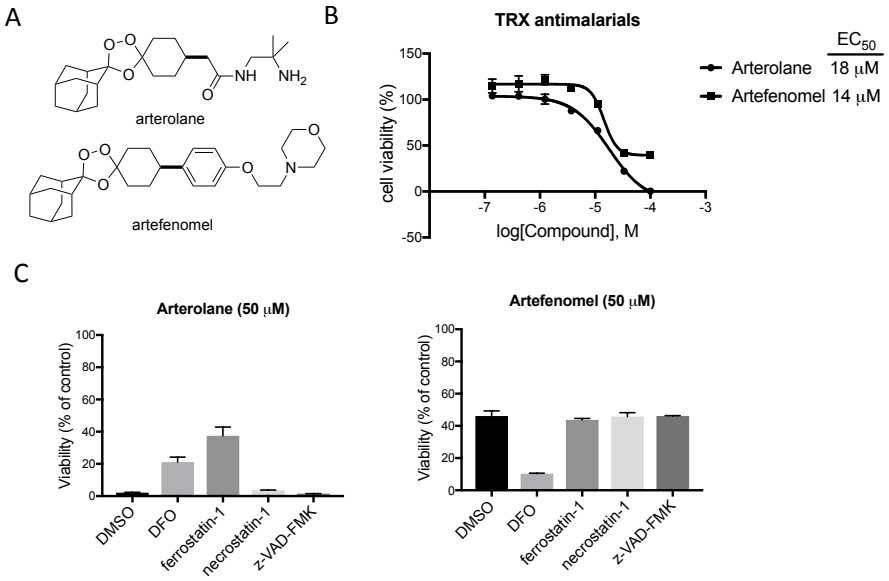


Figure S4. Cell killing by arterolane is characteristic of ferroptosis. A. Structures of arterolane and artefenomel. B. Dose response curves of antimalarials with calculated EC_{50}. Cell viability was measured after 24 h incubation in HT-1080 cells. C. Arterolane killing was partially rescued by DFO and ferrostatin-1, while artefenomel killing could not be rescued by any of the death supressors. HT-1080 cells were co-treated with antimalarials (50 μM) and death suppressors: DFO (100 μM), ferrostatin-1 (1 μM), necrostatin-1 (5 μM) and z-VAD-FMK (10 μM) or vehicle (DMSO). Cell viability was measured 24 h after incubation. Data are plotted as mean ± s.e.m. with two biological replicates.

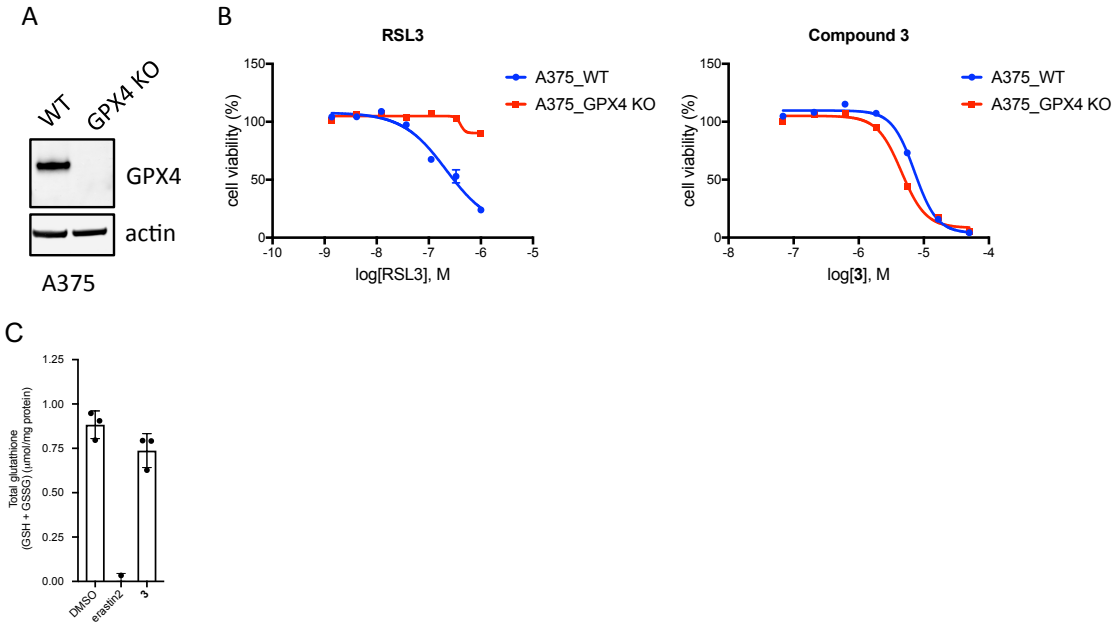


Figure S5. Compound **3** induced cell death is not dependent on GPX4 and does not deplete glutathione. A. Western blotting of A375 WT and GPX4 KO cells. B. Dose response curve of RSL3 and **3** in A375 WT and GPX4 KO cells. Cell viability was measured after 72 h incubation. C. Measurement of intracellular total glutathione levels of A549 cells treated with erastin2 (2 μM), or compound **3** (40 μM).

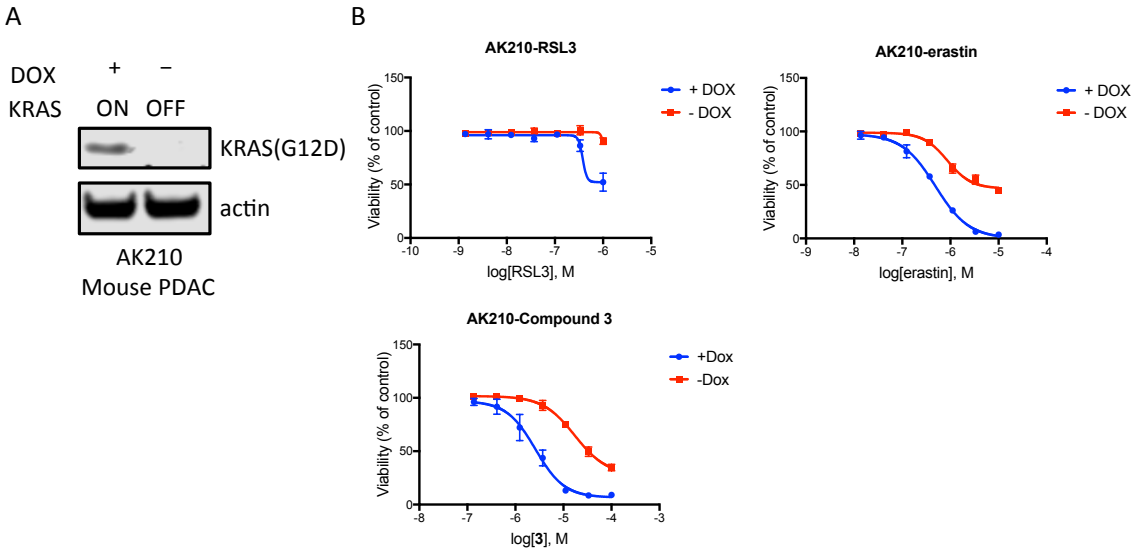


Figure S6. Compound **3** exhibits increased lethality in mouse PDAC cells expressing KRAS (G12D). A. Western blotting of mouse PDAC cells (AK210) with doxycycline-inducible KRAS (G12D) expression revealed KRAS (G12D) protein level after withdrawal of Dox for 72 h. B. Dose response curve of ferroptosis inducers in mouse PDAC cells with or without KRAS (G12D) expression. Cell viability was measured after 72 h drug incubation.

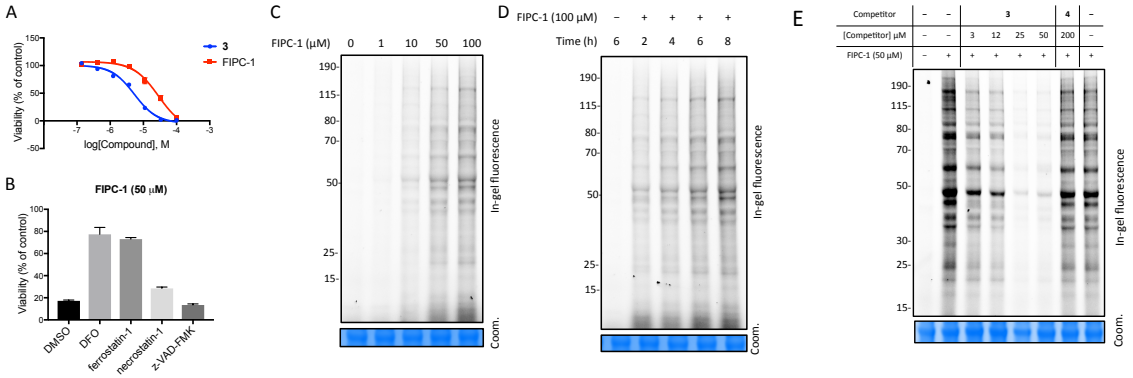


Figure S7. Probe FIPC-1 induces ferroptosis and exhibits iron-dependent, competable labelling of HT1080 proteomes. A. Dose response curve of **3** and FIPC-1 in HT-1080 cells. Cell viability was measured 24 h after incubation. B. DFO and ferrostatin-1 effectively rescue the cell death induced by FIPC-1. HT1080 cells were co-treated with FIPC-1 (50 μM) and a death suppressor: DFO (100 μM), ferrostatin-1 (1 μM), necrostatin-1 (5 μM), z-VAD-FMK (10 μM) or vehicle (DMSO). Cell viability was measured 24 h after incubation. C. FIPC-1 demonstrated concentration-dependent labeling as revealed by in-gel fluorescence. HT-1080 cells were incubated with FIPC-1 at indicated concentrations for 5 h, followed by cell lysis, CuAAC with TAMARA azide, SDS/PAGE, and in-gel fluorescence analysis. Coomassie blue staining was used as a loading control. D. Time course of FIPC-1 labeling events by in-gel fluorescence. E. Competition of FIPC1 labeling by compound **3** but not non-peroxidic control **4** as determined by in-gel fluorescence.

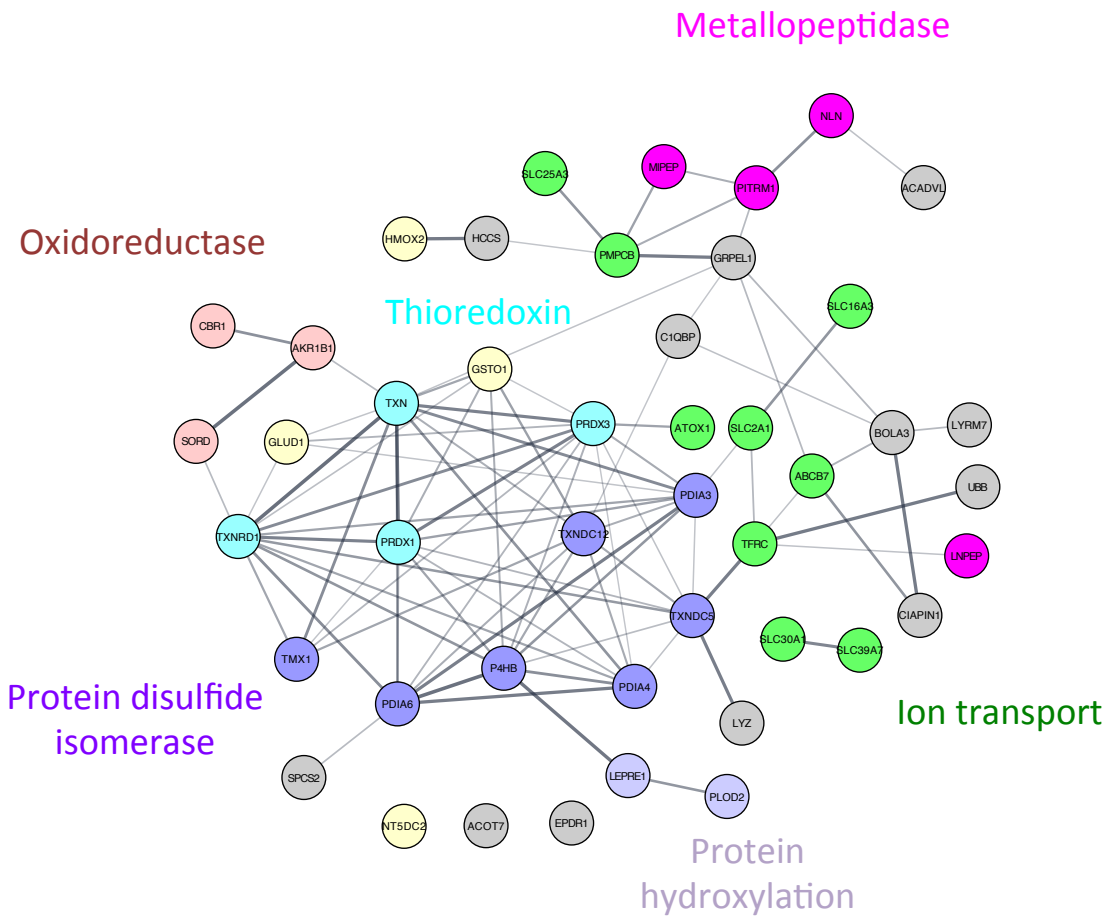


Figure S8. Protein interactome network of the enriched 45 proteins plotted by Cytoscape v3.7.2 with STRING and STRING enrichment plugin.[2,3]

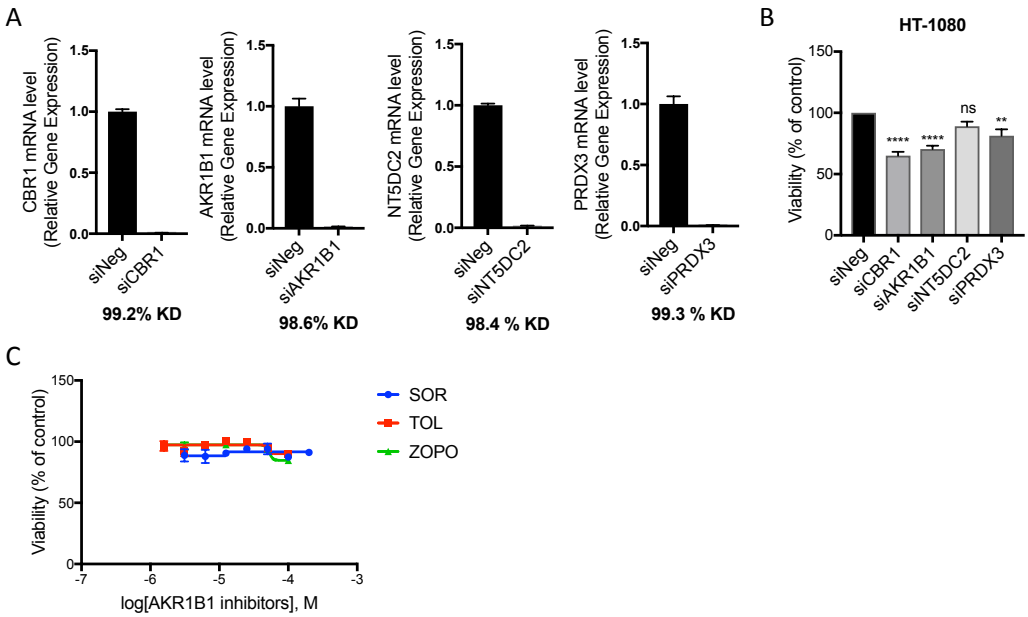


Figure S9. SiRNA knockdown and pharmacological inhibition of FIPC-1 targets. A. HT-1080 cells were transfected with siNeg or the corresponding siRNA pools for 72 h. mRNA level was calculated based on RT-qPCR. B. Individual siRNA knockdown of four target genes showed modest or not significant (NT5DC2) effect on cell viability. Cell viability was measured at 72 h post-transfection with siRNA pools. Data are normalized to siNeg control group and plotted as mean ± s.e.m with $n \geq 3$ biological replicates. One-way ANOVA multiple comparisons, ****$P \leq 0.0001$, ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, ns $P > 0.5$. C. AKR1B1 inhibitors (> 100 μM) did not trigger cell death in HT-1080 cells. Cell viability was measured after 24 h incubation. SOR: sorbinil, TOL: tolrestat, ZOPO: zopolrestat.

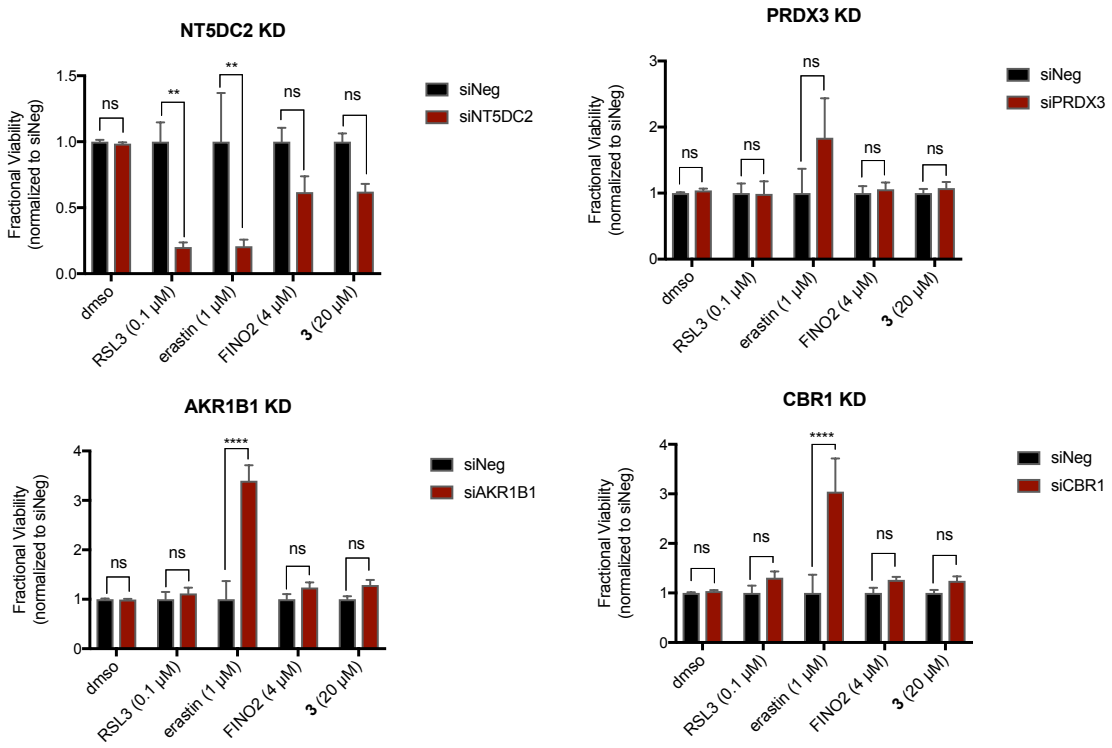


Figure S10. Target validation by knockdown of top 4 candidates from competitive proteomic profiling. Knockdown of *NT5DC2* significantly sensitized HT-1080 cells to RSL3 and erastin. Cells were treated with ferroptosis inducers at 48 h post-transfection with siRNA pools and viability was measured after 24 h drug incubation. Data are plotted as mean ± s.e.m, n = 4 biological replicates. Two-way ANOVA multiple comparisons, ****P ≤ 0.0001, ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05, ns P > 0.5.

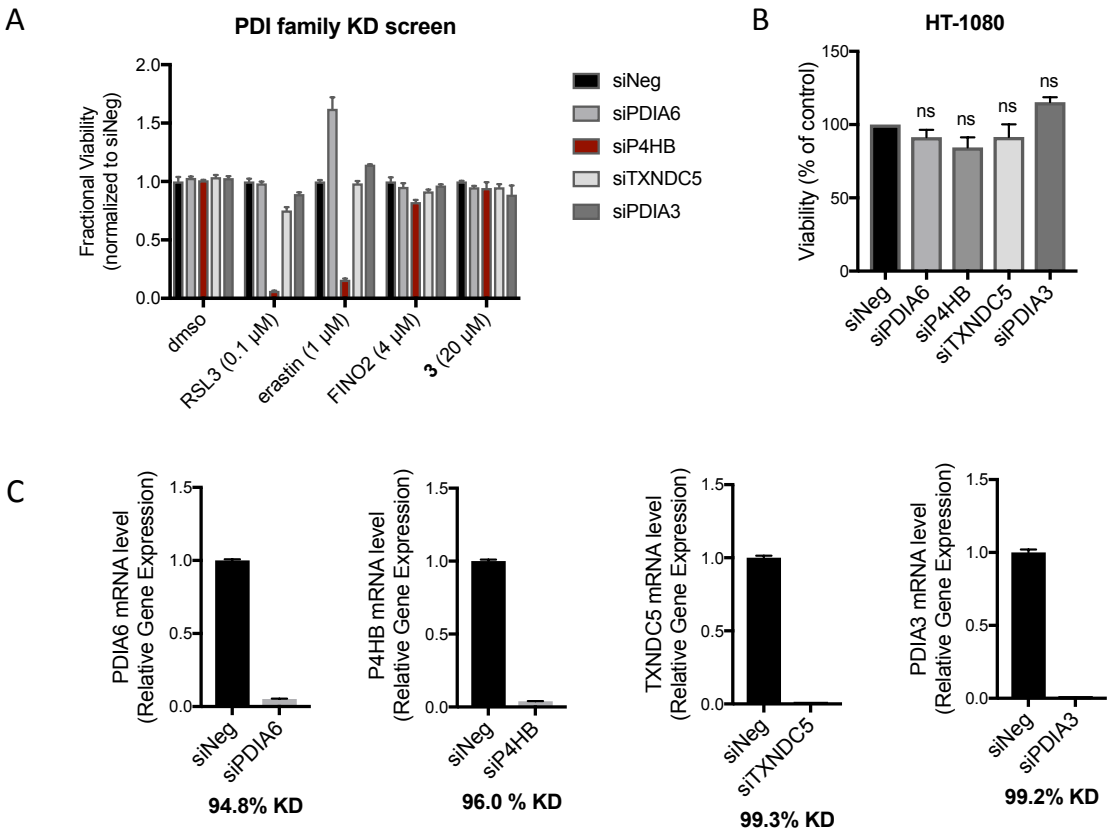


Figure S11. Effect of siRNA knockdown of PDI family members. A. siRNA knockdown of various PDIs revealed that only knockdown of *P4HB* sensitized HT-1080 cells to RSL3 and erastin. B. siRNA knockdown of 4 different PDIs has minimum impact on cell viability. Cell viability was measured at 72 h post-transfection with siNeg or the corresponding siRNA pools. Data are plotted as mean ± s.e.m, n ≥ 2 biological replicates. One-way ANOVA multiple comparisons, ns $P > 0.5$. C. HT-1080 cells were transfected with siNeg or the corresponding siRNA pools for 72 h. mRNA level was calculated based on RT-qPCR.

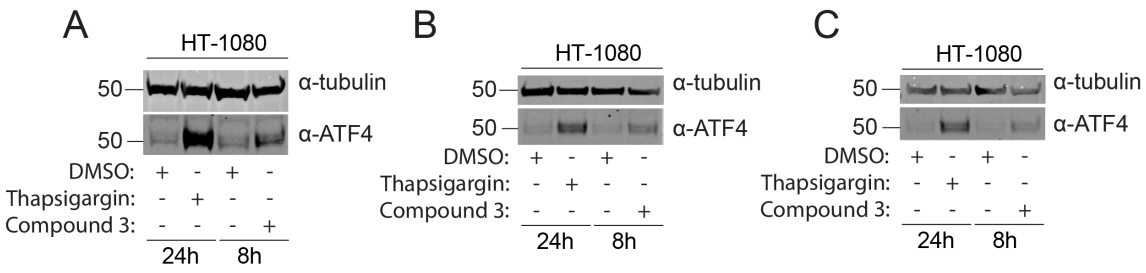


Figure S12. Western blot of HT-1080 cells treated with DMSO (vehicle), thapsigargin (100 nM), or Compound **3** (20 μM) for either 8h or 24h. Panels A-C represent three biological replicates.

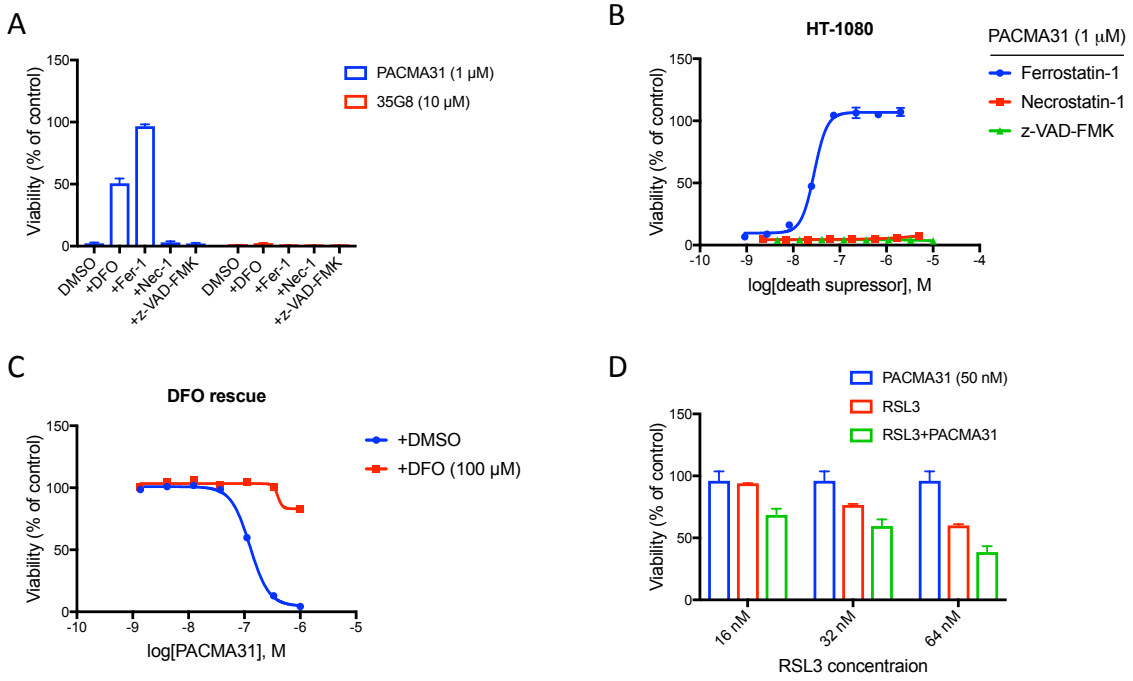


Figure S13. PDI inhibitor PACAM31 is a ferroptosis inducer and activates the unfolded protein response. A. Cell killing mediated by PACMA31 (1 μM) was partially or fully rescued by DFO (100 μM) or ferrostatin-1 (1 μM), but not by necrostatin-1 (5 μM) or z-VAD-FMK (10 μM). Viability at 24 h of HT-1080 cells co-treated with PDI inhibitors or death suppressor. Cell viability was measured 24 h after co-incubation. B. Viability at 24 h of HT-1080 cells co-treated with PACMA31 (1 μM) and increasing concentrations of ferrostatin-1, necrostatin-1, or z-VAD-FMK. C. DFO (100 μM) rescue cell death mediated by increasing concentrations of PACMA31 in HT-1080 cells. Cell viability was measured after 24 h of co-incubation. D. Viability at 24 h of HT-1080 cells co-treated with PACMA31 (50 nM) and increasing concentrations of RSL3. In A&D, data are plotted as mean ± s.e.m. with 2 biological replicates.

Methods and Materials

Cell culture and general materials. HT-1080 cell lines were obtained from ATCC and grown in MEM (HyClone) with glutamine and supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin and streptomycin mix (Pen/Strep, Gemini Bio-Products), and 1% nonessential amino acids (Lonza). A375 and A375 GPX4 KO cells[4] were obtained from Dr. M. Hangauer and Dr. F. McCormick (University of California, San Francisco), and grown in DMEM (HyClone) supplemented with 10% FBS and 1% Pen/Strep. Mouse PDAC cells (AK210) with doxycycline-inducible KRAS (G12D) expression system were obtained from Dr. R. Perrera (University of California, San Francisco) and maintained in RPMI (HyClone) supplemented with 10% FBS and 1% Pen/Strep in the presence of 1 μg/mL doxycycline (Fisher Bioreagents). All cells were maintained at 37 ºC in a humidified 5% CO_2 atmosphere. Chemical were purchased from commercially available sources unless noted otherwise: erastin (Cayman), 1S, 3R- RSL3 (Sigma-Aldrich), ferrostain-1 (Cayman), necrostatin-1 (Cayman), z-VAD(OMe)-FMK (Cayman), 35G8 (3-methyltoxoflavin, MCE), LOC14 (MCE), PACMA31 (TOCRIS), FAC (Ferric ammonium citrate, Sigma-Aldrich) DFO (Deferoxamine mesylate, Sigma-Aldrich), zopolrestat (Sigma-Aldrich), sorbinil (Sigma-Aldrich), tolrestat (Sigma-Aldrich).

Compound susceptibility testing. HT-1080 cells were seeded at 10,000 cells/well in 96-well clear bottom white plates (Greiner) and allowed to adhere overnight. The next day, cells were then treated with either vehicle (DMSO) or a dilution series of drug (in DMSO stock solution) for 24 h unless noted otherwise. Cell viability was determined by CellTiter-Glo (Promega) according to the manufacturer's protocol. Luminescence was read on a FlexStation 3 multi-mode microplate reader (Molecular Devices). Cell viability data were normalized to the DMSO treated condition. The dose response curves were plotted in mean ± sd and EC_{50} values were computed using Prism 7.0 (GraphPad).

Ferroptosis rescue assay. HT-1080 cells were seeded at 10,000 cells/well in 96-well clear bottom white plates (Greiner) and allowed to adhere overnight. For ferroptosis rescue assays, cells were replenished with fresh media containing a ferroptosis inducer and a dilution series of protective molecule (such as ferrostatin-1, necrostatin-1, z-VAD-FMK, or DFO) for 24 h. The final concentration of ferroptosis inducers was 10 μM FINO2, 20 μM FINO3, and 40 μM compound **3**. For iron chelator rescue assay, cells were co-treated with a dilution series of ferroptosis inducers and 100 μM of DFO for 24 h. In all cases, the cell viability was determined by CellTiter-Glo (Promega) as described above.

C11 BODIPY lipid peroxidation measurement. HT-1080 cells were seeded at 500,000 cells/well in 6-well plates and allowed to adhere overnight. The next day, cells were replenished with fresh media containing vehicle (DMSO), 10 μM of erastin, FINO2, FINO3 or compound **3** with or without DFO (50 μM) and incubated for 5 h at 37 °C. Cells were then trypsinized, washed once with HBSS, resuspended in HBSS buffer containing 2 μM C11 BODIPY 581/591 (Invitrogen) and incubated at 37 °C for 10 min. Cells were pelleted and resuspended in HBSS. Fluorescence intensity was measured on the FL1 channel with gating to record live cells only (gate constructed from DMSO treatment

group) using BD FACSCalibur Flow Cytometer. A minimum of 10,000 cells was analyzed per condition. Flow cytometry data was analyzed by FlowJo 10.6.

C11 BODIPY 581/591 imaging. HT-1080$^{PM\text{-}mTq2}$ cells were described previously[5] and grown in Dulbecco's Modified Eagle Medium, (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Gibco), 1X non-essential amino acids (Gibco) and 0.5 U/mL Pen/Strep (P/S, Gibco). Dimethyl sulfoxide (DMSO) and ferrostatin-1 were from Sigma-Aldrich. C11 BODIPY 581/591 (herein referred to as "C11") was from Molecular Probes. The day before the experiment, 150,000 HT-1080$^{PM\text{-}mTq2}$ cells/well were seeded into a 6-well plate (Corning) that had one 22 mm^2 no. 1.5 glass coverslip in each well. The following day, the cells were treated with compound **3** or **4** (each at 20 μM) and DMSO or ferrostatin-1 (1 μM). After 4.75 h, the medium was removed and the cells were labeled with C11 (5 μM) dissolved in HBSS and incubated at 37°C for 10 min. After 10 min, C11 was removed and fresh HBSS was put on to the cells. Each cover slip was inverted and mounted in 25 μL HBSS onto a glass microscope slide. Cells were imaged using a Zeiss Axio Observer microscope with a confocal spinning-disk head, PlanApoChromat 63×/1.4 NA oil immersion objective, and a Cascade II:512 electron-multiplying (EM) CCD camera (Photometrics) using the following excitation and emission/bandpass wavelengths: Non-oxidized C11: 561 nm and 593/40 nm; Oxidized C11: 488 nm and 520/35 nm; PM-mTq2: 405 nm and 440/40 nm. Images were processed in ImageJ 1.48v. Imaging was performed on two independent biological replicates per treatment condition and one representative replicate is shown.

Reduced glutathione measurement. A549^N cells were described previously[6] and grown in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS (Gibco) and 0.5 U/mL P/S (Gibco). The day before the experiment, 150,000 A549^N cells were seeded into 6-well dishes (Corning). Two wells were seeded per condition. 24 hours after seeding, cells were treated with DMSO (vehicle), erastin2 (compound 35MEW28[7]; 2 μM) or compound **3** (40 μM) for 6 h. Each pair of wells for each condition was subsequently washed with 1 mL HBSS each (Thermo Fisher Scientific) before being scraped into a total volume of 500 μL 1X MES (Cayman), then prepared for quantification of total intracellular glutathione (GSH) using an Ellman's reagent-based glutathione assay kit according to the manufacturer's protocol (Cayman). A glutathione standard curve was used to determine the GSH concentration in each sample, normalized to the total protein in each sample, which was calculated using the BCA assay (ThermoFisher Scientific). Three independent experiments were performed for each condition.

In-gel fluorescence analysis. HT-1080 cells were grown to ~90% confluence in 6-well plates. The growth media was removed and the cells were replenished with fresh media containing vehicle (DMSO) or FIPC-1 (as DMSO stock solution) at indicated concentrations and time at 37 °C. For competition experiment, cells were pre-incubated with vehicle (DMSO), ferrostatin-1 (10 μM) or compound **3** (5, 15, 50 μM) for 0.25 h at 37 °C, followed by addition of FIPC-1 (50 μM) for 5 h. After the treatment, the media was removed and the cells were washed with ice-cold PBS. Cells were then harvested by cell scrapers and the cell pellets were snap freeze by liquid nitrogen and stored at −80 °C. The pellet was thawed on ice and resuspended in 100 μL of NP40 lysis buffer (100 mM HEPES,

pH 7.5, 0.1% NP-40, 150 mM NaCl) with EDTA-free protease inhibitor cocktail (Roche). The lysate was incubated on ice for 30 min and centrifuged at 14,000 × g for 10 min at 4 °C. The protein concentrations were measured from each of the supernatant sample by BCA assay (Pierce) and adjusted to 1 mg/mL. CuAAC was performed at a final concentration of 25 μM TAMRA-azide (Click Chemistry Tools), 1 mM Tris(2-carboxyethyl)phosphine (TCEP, Thermo-Scientific), 100 μM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, Click chemistry Tools), and 1 mM $CuSO_4$ (Sigma-Aldrich) in a total volume of 50 μL. The reaction was performed at RT for 1 h in the dark before termination by addition of 1 mL ice-cold MeOH and placed in −80 °C for 1 h. The precipitated proteins were centrifuge at 14,000 x g for 5 min at 4 °C and the protein pellet were wash once more with ice-cold MeOH. Solubilize the precipitated protein pellet in 50 μL of 1× Laemmli sample buffer (Bio-Rad) supplemented with β-mercaptoethanol and boiled for 5 min. 25 μL of the samples were loaded and resolved on a 4-12% Bis-Tris protein gel (Invitrogen). Fluorescence was visualization by ChemiDoc imager (Bio-Rad) and the image was displayed as gray scale. Following fluorescence imaging, total protein was visualized by Coomassie stain as a loading control.

Iron conditioning. For iron conditioning experiments, cells were exposed to 500 μM ferric ammonium citrate (FAC, stock solution prepared in deionized water) or 300 μM iron chelator DFO (as DMSO stock solution) in complete growth medium for 2 h. For cells exposed to FAC, cells were then washed with PBS to remove any extracellular iron, and treated with FIPC-1 at indicated concentrations for 5 h. For cells pretreated with DFO, cells were continually exposed to DFO to prevent the cells from mobilizing iron stores and replenishing the labile Fe^{2+} pool during the course of treatment with FIPC-1. Post-treatment cells were washed with ice-cold PBS and followed by cell lysis, conjugation with TAMARA-azide via CuAAC, and in-gel fluorescence analysis as described above.

Target labeling, affinity purification and on-bead digestion. HT-1080 cells were grown to 90% confluence in 15-cm plates and incubated with fresh growth media containing vehicle (DMSO) or probe FIPC-1 (final concentration at 50 μM) for 5 h. For competition experiment, cells were pre-incubated with vehicle (DMSO) or compound **3** (at 5, 15, 50 μM) for 0.25 h at 37 °C, followed by addition of FIPC-1 (50 μM) for 5 h. Cells were then washed with cold PBS and harvested using cell scrapers. The cell pellets were resuspended in 500 μL of NP40 lysis buffer (100 mM HEPES, pH 7.5, 0.1% NP-40, 150 mM NaCl) with EDTA-free protease inhibitor cocktail (Roche). Cells were briefly sonicated in a bath sonicator and incubated on ice for 30 min before centrifugation at 5,000 × g at 4 °C for 15 min. Protein concentrations of cell lysates were adjusted to 2 mg/mL (total 1 mg of protein) and the lysates were subjected to CuAAC with 100 μM biotin-PEG3-azide (click chemistry tool), 1 mM TCEP, 100 μM TBTA (Click chemistry tool), and 1mM $CuSO_4$ (Sigma-Aldrich) at final concentration in a total volume of 0.5 mL and incubated in the dark for 1 h. 1.5 mL of ice-cold MeOH were added to the reaction mixtures and the proteins were allowed to precipitate at −20 °C overnight. Precipitated proteins were collected by centrifugation at 6,500 × g for 5 min and the pellets were washed twice with ice-cold MeOH. The pellets were then air-dried and resolubilize in 1 mL SDS buffer (1.2% w/v SDS in PBS), and the samples were heated at 70 °C for 5 min, then centrifuge at 6,500 × g for 5 min at RT. The resulting clear supernatants were incubated with Pierce Neutravidin

agarose (Thermo Scientific #29200) pre-equilibriumed with 5 mL PBS (final concentration of SDS is 0.2%) for 1 h at RT. The beads were further washed with 0.2% SDS PBS buffer, 1M NaCl containing PBS buffer, and lastly 50 mM ammonium bicarbonate buffer with 2 M Urea. The beads were transferred to clean eppendorf tubes and resuspend the beads with 0.5 mL 50 mM ammonium bicarbonate buffer with 2 M Urea supplemented with DTT (final concentration 9.75 mM) and incubated on a rotator at RT for 40 min, followed by addition of iodoacetamide (final concentration 20 mM) and incubated at RT for a further 30 min. The beads were washed twice with 50 mM of ammonium bicarbonate buffer and then resuspended in 50 mM of ammonium bicarbonate with 1 M Urea supplemented with MS grade trypsin protease (Pierce), and incubated at 37 ^{o}C for 16 h. After digestion, samples were acidified with 5% formic acid, and peptides recovered by affinity purification using OMIX C18 100 µL tips (Agilent Technologies) as described by the manufacturer. Peptides were eluted in 2 x 40 µL 50% MeCN with 0.1% formic acid, and solvent evaporated in preparation for TMT labeling.

TMT labeling. Dried samples were labeled according TMT 6-plex kit instructions (ThermoFisher Scientific, #90061), with minor modifications. Shortly, peptides were resuspended in 10 µL 50 mM TEAB (triethyl ammonium bicarbonate). TMT reagents were resuspended in 41 µL acetonitrile per vial, and 20 µL of this solution were added to the individual samples to be labelled. After incubating for 1 h at 22 ^{o}C, reactions were quenched by adding 4 µL 5% hydroxylamine and incubated for additional 15 min. After that, the labeling reactions were combined, partially evaporated to close to 5 µL, diluted in 60 µL 0.1% formic and desalted using a ZipTip C18 (Millipore) as indicated by the manufacturer. Peptides were eluted in 2 x 7 µL aliquots of 50% MeCN with 0.1% formic acid, dried and resuspended in 2.5 µL 0.1% formic acid for mass spectrometry analysis

Mass spectrometry analysis. Peptides were separated using a 75 µm x 50 cm PepMap RSLC C18 EasySpray column (Thermo Scientific) using 3-hour MeCN gradients (2–30% in 0.1% formic acid), for analysis in a Orbitrap Lumos Fusion (Thermo Scientific) in positive ion mode. MS spectra were acquired between 375 and 1500 m/z with a resolution of 120000. For each MS spectrum, multiply charged ions over the selected threshold (2E4) were selected for MS/MS in cycles of 3 seconds with an isolation window of 0.7 m/z. Precursor ions were fragmented by HCD using stepped relative collision energies of 30, 35 and 40 in order to ensure efficient generation of sequence ions as well as TMT reporter ions. MS/MS spectra were acquired in centroid mode with resolution 50000 from m/z=110. A dynamic exclusion window was applied which prevented the same m/z from being selected for 30s after its acquisition.

Peptide and protein identification and TMT quantitation. Peak lists were generated using PAVA in-house software.[8] All generated peak lists were searched against the human subset of the SwissProt database (SwissProt.2017.11.01), using Protein Prospector[9] with the following parameters: Enzyme specificity was set as trypsin, and up to 2 missed cleavages per peptide were allowed. Carbamidomethylation of cysteine residues, and TMT labeling of lysine residues and N-terminus of the protein were allowed as fixed modifications. N-acetylation of the N-terminus of the protein, loss of protein N-terminal methionine, pyroglutamate formation from peptide N-terminal glutamines, oxidation of

methionine were allowed as variable modifications. Mass tolerance was 10 ppm in MS and 30 ppm in MS/MS. The false positive rate was estimated by searching the data using a concatenated database which contains the original SwissProt database, as well as a version of each original entry where the sequence has been randomized. A 1% FDR was permitted at the protein and peptide level. For quantitation only unique peptides were considered; peptides common to several proteins were not used for quantitative analysis. Relative quantization of peptide abundance was performed via calculation of the intensity of reporter ions corresponding to the different TMT labels, present in MS/MS spectra. Intensities were determined by Protein Prospector. Summed intensity on each TMT channel for all identified spectra were used to normalize individual intensity values. Relative abundances were calculated as ratios vs the average intensity levels in control samples (non-probe, or probe with no competitor, as indicated). For total protein relative levels, peptide ratios were aggregated to the protein levels using median values of the log2 ratios. In competition curves for individual proteins, ion intensities in individual experiments at each concentration of the competitor were normalized from 1 ("probe" channel) to 0 ("non probe" channel).

siRNA Transfection. On TARGETplus small interfering RNA (siRNA) SMARTpools targeting each gene were purchased from Dharmacon (Horizon Discovery). A ON TARGETplus non-targeting siRNA was used as a negative control. Reverse transfection on HT-1080 cells was performed based on the previously described method with modifications.[10] In brief, 2 μL of 10μM siRNA solution (10 nM siRNA final concentration) and 6 μL of lipofectamine RNAiMAX reagent (Invitrogen) were diluted with 0.5 mL of Opti-MEM (Invitrogen) respectively, and the resulting DNA and siRNA dilutions were mixed and incubated for 15 min at 37 C in a tube. Following incubation, 200,000 HT-1080 cells suspended in 1 mL of 2X serum-containing media were transferred to each tube to prepare the master mix. The resulting cell mixtures were reseeded into 96-well format. The cells were replenished with fresh culture media after 24 h. For viability measurements, cells were added CellTiter-Glo reagents at 72 h post-transfection. For drug treatment experiments, transfected cells were treated with drugs at indicated concentrations at 48 h post-transfection, incubated for 24 h, and the cell viability measurements were performed as described above.

Gene expression analysis by RT-qPCR. HT-1080 cells transfected with siRNA from 12-well format were harvest at 72 h post-transfection. RNA was extracted using Qiagen RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription reactions were conducted using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814). Quantitative PCR reactions were performed using Maxima SYBR Green qPCR Master Mix (Thermo Scientific, # K0251). The samples in triplicates per condition were analyzed on a PikoReal Real Time PCR system (Thermo Fischer). Differences in mRNA levels between control and experimental conditions were calculated using the ΔΔCt method with ACTB as an internal reference gene. Primers for qPCR were designed with Primer Blast.

Western blotting.

GPX4 KO and KRAS(G12D) Induction Experiments. Cells were washed with PBS and harvested by cell scrapers from 12-well format. The collected cell pellets were resuspended in 30 μL cell lysis buffer (cell signaling technology, #9803) with EDTA-free protease inhibitor cocktail (Roche). Cells were incubated in lysis buffer for 20 min on ice and the resulting lysates were centrifuged at 14,000 × g for 10 min at 4 ºC to clear the cell debris. The protein concentrations were measured from each of the supernatant sample by BCA assay (Pierce) and normalized for total protein content with lysis buffer and subsequently denatured in Laemmli loading buffer with β-mercaptoethanol (BME) at 95 °C for 5 min. 15 μg protein from each sample was then loaded onto Bolt 4–12% Bis-Tris Plus Gels (Invitrogen) and run at 190 V for 35 min, then transferred to an PVDF membrane using iBlot 2 Gel Transfer Device (Invitrogen). The membrane was blocked with Odyssey blocking buffer (TBS) (Li-COR) for 1 h at RT then incubated with the indicated antibodies in Odyssey buffer containing 0.1% Tween-20 overnight at 4 °C. The blot was then washed three times with TBS-T and incubated with Azure Spectra 700 and 800 secondary antibodies in Odyssey blocking buffer containing 0.1% Tween-20 and 0.01% SDS for 1 h at RT. The blot was washed four times with TBS-T for 5 min and once with TBS then imaged for fluorescent signal on an Odessey Classic Infrared Imaging System. Antibodies were obtained from commercially available sources: KRAS (G12D mutant specific) antibodies (Cell signaling Technology, #14429S). GPX4 antibodies (Invitrogen, #MA5-32827), Beta Actin mouse mAb (Cell Signaling Technology, #3700S). Primary antibodies were used at a 1:2,000 dilution; secondary antibodies were used at a 1: 10,000 dilution.

ATF4 Induction Experiment. On day 1, 700,000 HT-1080 cells were seeded into 10 cm dishes (Cat# CC7682-3394, USA Scientific, Ocala, FL) for each condition. 24 h later, cells were treated with DMSO (vehicle), compound **3** (20 μM), or thapsigargin (100 nM). After 8 h, the media was removed from cells treated with compound **3** and DMSO, cells were washed with 2 mL 1x PBS, and then scraped off the plate into 1.5 mL 1x PBS. Controls treated with thapsigargin or DMSO were harvested as described above following 24 h of treatment. Cell suspensions were transferred into a 1.5 mL microcentrifuge tube then centrifuged at 3,000 rpm for 5 min. After discarding the supernatant, the cell pellet was lysed in 100 μL 9 M urea. Lysates were sonicated [(1 s on, 1 s off, 60 % amplitude) x 10 cycles] and centrifuged (13,300 rpm, 15 min, room temperature). The supernatant was transferred to a new 1.5 mL tube. Total cell lysate protein concentration was measured by BCA assay kit (Thermo Fisher Scientific, Cat# 23252) using a standard BSA curve. 20 μg of protein from each sample were mixed with 4x Bolt LDS Sample Buffer (Cat# B0007) and 10x Bolt Sample Reducing Agent (Cat# B0009) (Life Technologies), and 9M urea was added to total each sample volume to 30 μL. Samples were heated in a thermocycler to 95°C for 5 min then loaded onto a Bolt 4-12% Bis-Tris Plus Gel (Cat# NW04120BOX) (Life Technologies). Proteins from the gel were transferred to a nitrocellulose membrane using an iBlot2 transfer stack (Life Technologies). The membrane was blocked with Intercept Blocking Buffer (Cat# 927-70001, LI-COR Biotechnology, Lincoln, NE) (1 h, RT) and then incubated in primary antibody mixture (16 h, 4°C). Primary antibodies used were α-ATF4 (Cat#11815S, Cell Signaling Technology; 1:1000 dilution) and α-tubulin (Cat# MS581P1, Millipore Sigma, Billerica, MA; 1:1000 dilution). The membrane was

washed 3x in TBST and then incubated in secondary antibody mixture (1 h, RT). Secondary antibodies used were donkey α-rabbit (Cat# 925-32213, LI-COR Biotechnology, Lincoln, NE, 1:15,000 dilution) and donkey α-mouse (Cat# 926-68022, LI-COR, 1:15,000 dilution), and the secondary antibody buffer was Intercept Blocking Buffer (Cat# 927-70001 , LI-COR Biotechnology, Lincoln, NE). The membrane was washed 3x in TBST for 5 min each cycle, and then scanned on an Odyssey CLx Imaging System (LI-COR). The experiment was repeated three times.

Lipidomic Analysis of Compound 3

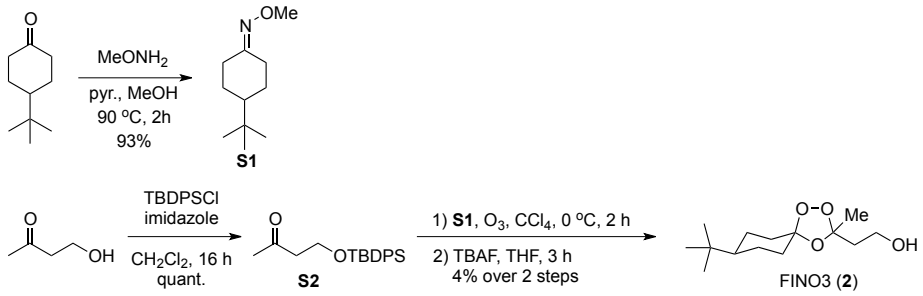
HT-1080 cells were grown to ~90% confluence in 15-cm culture dishes. The growth medium was removed and the cells were replenished with fresh media containing vehicle (DMSO) or compound **3** (50 μM), in 6 replicates for each condition, and incubated at 37 ^{o}C for 5.5 h. The media were then removed, and cells were washed with cold PBS once and then trypsinized. Neutralized trypsin with culture media and centrifuged at 1000 rpm for 5 min. The media was removed and the cell pellets were resuspended in cold PBS. An aliquot of cell suspension from each sample was taken for cell counting using Countess II automated cell counter (Invitrogen). Next, the cell suspension was centrifuged at 1000 rpm for 5 min at RT, and the PBS was removed. The frozen cell pellets (around 10 million cells in each sample) were snap frozen in liquid nitrogen and stored at −80 ^{o}C. The frozen pellets were then submitted to West Coast Metabolomics Center (UC Davis) for lipid extraction and complex lipid analysis by CSH-QTOF MS/MS. In brief, lipids were extracted from cells with methanol and methyl tert-butyl ether, both containing a cocktail of lipid standards (Avanti Polar lipids, Alabaster, USA).[11] Water was subsequently added for phase separation. After concentrating extracts to complete dryness, samples were reconstituted prior to LC-MS analysis in 110 μL of methanol:toluene (90:10, v/v) with 50 ng/mL CUDA standard.[12] All measurements were carried out on an Agilent 6530a QTOF instrument. For positive mode, 0.4 μL of diluted samples were injected. For negative mode, 5 μL of diluted samples were injected. Samples were separated on a Waters Acquity UPLC CSH C18 column (100 × 2.1 mm; 1.7 μm) coupled to an Acquity UPLC CSH C18 VanGuard precolumn (5 × 2.1 mm; 1.7 μm). The LC-MS/MS data was analyzed by MS-DIAL software.[13] Lipids were quantified by peak height and the raw peak heights were normalized to the cell count of each sample before data processing. Statistical analysis was carried out using Student's t-Test to determine differentially represented normalized mass peak between the DMSO- and compound **3**-treated samples (n = 6 for each condition). Lipids whose normalized mass peaks changed significantly ($P < 0.05$) were plotted using Prism. One low abundance outlier lipid was removed from the final plot. The complete dataset is provided as a supplemental file.

Synthetic Procedures

General Procedures: Reactions were magnetically stirred unless otherwise indicated. Air and/or moisture sensitive reactions were carried out under an argon atmosphere using anhydrous solvents from commercial suppliers. Air and/or moisture sensitive reagents were transferred via syringe or cannula and were introduced into reaction vessels through rubber septa. Reaction product solutions and chromatography fractions were concentrated by rotary evaporation at room temperature. Thin phase chromatography was performed on EMD precoated glass-backed silica gel 60 F-254 0.25 mm plate.

Materials: All chemical reagents and solvents used were purchased from commercial sources, such as Sigma-Aldrich and Fisher Scientific. Anhydrous DMF, dichloromethane and tetrahydrofuran (EMD Drisolv) were used without further purification. Known compounds FINO2 (**1**)[14], **5**[15], and intermediates **S3**,[16] **S5**,[16] and **S7**,[17] were prepared using the reported procedures.

Instrumentation: ^{1}H NMR spectra were recorded on a Varian INOVA-400 400 MHz spectrometer. Chemical shifts are reported in δ units (ppm). NMR spectra were referenced relative to residual NMR solvent peaks. Coupling constants (*J*) are reported in hertz (Hz). When an NMR resonance is unambiguously attributable to a minor diastereomer this is indicated as "(minor)", but otherwise is not. Column chromatography was performed on Silicycle Sili-prep cartridges using a Biotage Isolera Four automated flash chromatography system. LC/MS and compound purity were determined using Waters Micromass ZQTM, equipped with Waters 2795 Separation Module and Waters 2996 Photodiode Array Detector. Separations were carried out with an XTerra® MS C18, 5μm, 4.6 x 50 mm column, at ambient temperature (unregulated) using a mobile phase of water-methanol containing a constant 0.1 % formic acid.



Scheme S1. Synthesis of FINO3 (**2**).

4-(*tert*-butyl)cyclohexanone *O*-methyl oxime (S1). To a sealed tube was added 4-tert-butylcyclohexanone (4 g, 0.026 mol, 1.0 equiv.), methanol (30 mL), methoxylamine hydrochloride (3.25g, 0.039 mol, 1.5 equiv.), and pyridine (4.19 ml, 0.052 mol, 2.0 equiv.). The reaction mixture was stirred at RT until all starting materials dissolved. The tube was sealed with a teflon cap equipped with a O-ring and heated in a preheated oil bath at 90 °C for 2 h. The reaction mixture was allowed to cool to RT, and the solvent was removed to obtain a yellow residue. The crude product was diluted into ethyl acetate and washed with water (30 mL), 10% $KHSO_4$ solution (30 mL x 3), and brine (10 mL). The organic phase was separated and dried over anhydrous Na_2SO_4, filtered, and concentrated to afford the desired compound as pale yellow oil (4.4 g, 93%) that was used in the next step without further purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 3.81 (s, 3H), 3.26 (tdd, *J* = 2.3, 4.2, 14.5 Hz, 1H), 2.45 - 2.39 (m, 1H), 2.09-2.11 (m, 1H), 1.93 - 1.87 (m, 2H), 1.67 (dt, *J* = 5.4, 13.9 Hz, 1H), 1.27 - 1.09 (m, 3H), 0.87 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 160.3, 60.9, 47.5, 32.4, 32.0, 27.7, 27.5, 26.4, 24.9.

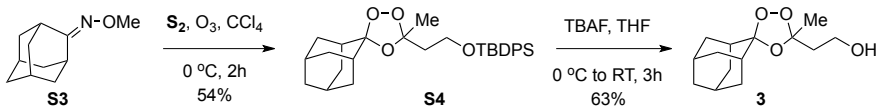
4-((*tert*-butyldiphenylsilyl)oxy)butan-2-one (S2). To a round bottom flask charged with 4-hydroxy-2-butanone (1 g, 0.011 mol, 1.0 equiv.) in dichloromethane (25 mL) was added imidazole (1.54 g, 0.022 mol, 2.0 equiv.). The mixture was cooled in an ice bath and *tert*-butyldiphenylsilylchloride (3.09 mL, 0.012 mol, 1.05 equiv.) was added dropwise. The reaction mixture was allowed to warm slowly to RT and stirred for 16 h. The reaction mixture was then diluted with water and extracted with dichloromethane (20 mL x 2). The combined organic phases were then washed with brine (10 mL), dried over

anhydrous Na_2SO_4, filtered, and concentrated. The crude residue was purified on a 120 g silica gel cartridge with gradient elution (2–8% EtOAc in hexanes) to obtain the desired product as colorless oil (3.87 g, quant.).

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 7.80 - 7.57 (m, 4H), 7.54 - 7.27 (m, 6H), 3.97 (t, *J* = 6.2 Hz, 2H), 2.66 (t, *J* = 6.2 Hz, 2H), 2.21 (s, 3H), 1.06 (m, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 207.9, 135.5, 133.4, 129.7, 127.7, 59.7, 46.3, 30.7, 26.7, 19.1.

2-(8-(*tert*-butyl)-3-methyl-1,2,4-trioxaspiro[4.5]decan-3-yl)ethanol (FINO3, 2). A round bottom flask was charged with ketone **S2** (1.0 g, 3.1 mmol, 1.0 equiv.), carbon tetrachloride (40 mL), and oxime **S1** (0.84 g, 4.6 mmol, 1.5 equiv) and cooled at 0 °C while ozone gas was carefully bubbled into the solution for 2 h. At this time the reaction was judged incomplete and an additional portion of oxime **S1** (0.26 g, 1.41 mmol, 0.5 equiv.) was added, followed by ozone gas for another 2 h. The reaction mixture was then purged with O_2 for 10 min followed by argon for 15 min. The reaction mixture was concentrated and purified on a 40 g column of silica gel with gradient elution (2–10% EtOAc in hexanes) to obtain the trioxolane product as yellow oil (480 mg) that was used in the next step directly. To a round bottom flask containing the crude product was added THF (1 mL). After cooling in an ice bath, the mixture was treated with 1M tetrabutyl ammonium fluoride solution in THF (2.9 ml, 2.9 mmol, 3.0 equiv.). The reaction mixture was stirred at RT for 4 h and then diluted with brine (5 mL) and extracted with EtOAc (5 mL x 2). The organic layers were combined, dried over Na_2SO_4, filtered, concentrated. The crude residue was then purified on a 25 g column of silica gel with gradient elution (5–20% EtOAc in hexanes) to obtain the desired product as a colorless oil (32 mg, 4% over 2 steps).

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 3.89 - 3.75 (m, 2H), 2.48-2.23 (br, 1 H), 2.10 - 1.98 (m, 2H), 1.96 - 1.80 (m, 4H), 1.62 - 1.56 (m, 2H), 1.51 (s, 3H), 1.41 - 1.23 (m, 2H), 1.03 (tt, *J* = 3.0, 12.1 Hz, 1H), 0.68 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 110.2, 109.9, 58.7, 47.1, 39.4, 34.4, 34.2, 32.3, 27.6, 24.6, 24.5, 23.6. LC-MS (ESI) calculated for $C_{14}H_{26}O_4$ *m/z* $[M+Na]^+$ =281.17, found 281.00.



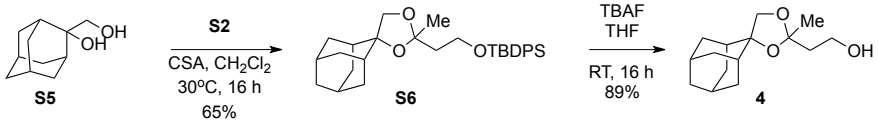
Scheme S2. Synthesis of compound **3**.

***tert*-butyl(2-(5'-methylspiro[adamantane-2,3'-[1,2,4]trioxolan]-5'-yl)ethoxy)diphenylsilane (S4).** To a round bottom flask charged with oxime **S3** (0.411 g, 2.29 mmol, 1.50 equiv.) in carbon tetrachloride (20 mL) was added a solution of ketone **S2** (0.500 g, 1.53 mmol, 1.0 equiv.) in carbon tetrachloride (10 mL). The reaction mixture was cooled in an ice bath while ozone was bubbled through the reaction mixture for 1 h. An additional portion of oxime **S3** (0.150 g, 0.837 mmol, 0.55 equiv.) was then added and ozone was bubbled through the reaction for another 1 h. The reaction flask was then purged with O_2 for 10 min followed by argon for 15 min and the mixture was then concentrated to oil. The crude product was purified on a 40 g column of silica gel with gradient elution (0–5% EtOAc in hexanes) to obtain the desired product as a colorless oil/white foam (406 mg, 54%).

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 7.80 - 7.57 (m, 4H), 7.47 - 7.34 (m, 6H), 3.89 - 3.76 (m, 2H), 2.05 - 1.61 (m, 16H), 1.48 (s, 3H), 1.05 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 135.6, 133.7, 129.6, 127.6, 111.7, 108.9, 60.0, 40.6, 36.8, 36.3, 36.0, 34.8, 34.7, 33.2, 27.1, 26.8, 26.4, 23.7, 19.1. LC-MS (ESI) calculated for $C_{30}H_{40}O_4Si$ *m/z* $[M+Na]^+$ = 515.26, found 515.21.

2-(5'-methylspiro[adamantane-2,3'-[1,2,4]trioxolan]-5'-yl)ethanol (3). A round bottom flask charged with intermediate **S4** (0.340 g, 0.69 mmol, 1.0 equiv.) was cooled in an ice bath and treated with a 1M solution of tetrabutyl ammonium fluoride solution in THF (3.450 ml, 3.5 mmol, 5.0 equiv.). The reaction mixture was allowed to warm to RT and stirred for 2.5 h. The reaction mixture was then diluted with EtOAc, washed with water (10 mL x 2), brine (5 mL x 1), and the organic phase dried over anhydrous Na_2SO_4, filtered, and concentrated. The crude residue was purified on a 12 g column of silica gel with gradient elution (3–20% EtOAc in hexanes) to obtain the desired product as colorless oil (110 mg, 63%).

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 3.87 - 3.74 (m, 2H), 2.45 (br, 1H), 2.11 - 1.92 (m, 8H), 1.88 - 1.69 (m, 8H), 1.50 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 112.3, 109.9, 58.7, 39.5, 36.6, 36.3, 36.0, 34.9, 34.8, 34.8, 34.7, 26.7, 26.3, 23.7. LC-MS (ESI) calculated for $C_{14}H_{22}O_4$ *m/z* $[M+Na]^+$ = 277.14, found 276.97.



Scheme S3. Synthesis of **4**.

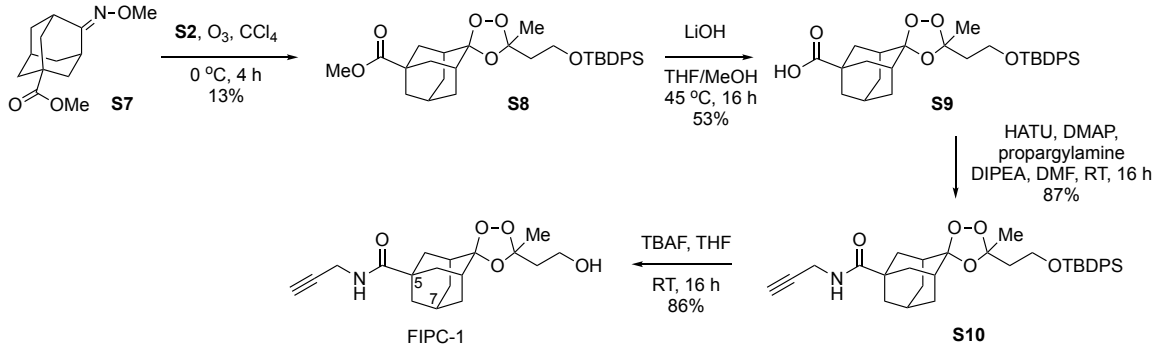
***tert*-butyl(2-(2'-methylspiro[adamantane-2,4'-[1,3]dioxolan]-2'-yl)ethoxy)diphenylsilane (S6).** To a round bottom flask charged with diol **S5** (75 mg, 0.4 mmol, 1.0 equiv.) and ketone **S2** (134 mg, 0.4 mmol, 1.0 equiv.) in dichloromethane (4 mL) was added camphor sulfonic acid (24 mg, 0.1 mmol, 0.3 equiv.). The reaction mixture was heated at 30 °C for 16 h and then cooled to RT. The reaction mixture was then diluted with dichloromethane (10 mL), washed with saturated $NaHCO_3$ solution (10 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated. The crude residue was purified on a 12 g column of silica gel with gradient elution (2–10% EtOAc in hexanes) to afford the product as colorless oil (135 mg, 65%).

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 7.73 - 7.66 (m, 4H), 7.46 - 7.36 (m, 6H), 3.89 - 3.78 (m, 3H), 3.73 (d, *J* = 8.5 Hz, 1H), 2.14 (br d, *J* = 11.9 Hz, 1H), 2.06 - 1.87 (m, 3H), 1.87 - 1.43 (m, 12H), 1.35 (s, 3H), 1.06 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 135.6, 133.9, 129.5, 127.6, 108.9, 84.7, 72.3, 60.6, 42.9, 37.4, 37.3, 37.0, 35.9, 35.8, 33.5, 33.4, 26.9, 26.8, 26.7, 26.3, 19.1. LC-MS (ESI) calculated for $C_{31}H_{42}O_3Si$ *m/z* $[M+Na]^+$ = 513.28, found 513.30.

2-((2'-methylspiro[adamantane-2,4'-[1,3]dioxolan]-2'-yl)ethanol (4). A round bottom flask charged with **S6** (105 mg, 0.2 mmol, 1.0 equiv.) and THF (1 mL), was cooled at in an ice bath and a solution of 1 M tetrabutyl ammonium fluoride solution in THF (1.070 ml, 1.1 mmol, 5.0 equiv.) was added dropwise. The reaction mixture was slowly warmed up RT and stirred for 16 h. The reaction mixture was then diluted with EtOAc (5 mL), washed

with water (5 mL), brine (5 mL) and the organic layer was dried over anhydrous Na_2SO_4, filtered, concentrated. The crude residue was purified on a 12 g column of silica gel with gradient elution (10–30% EtOAc in hexanes) to obtain the product as colorless oil (48 mg, 89%).

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 3.95 (d, *J* = 8.8 Hz, 1H), 3.87 (d, *J* = 8.8 Hz, 1H), 3.82 - 3.69 (m, 2H), 2.87 (br s, 1H), 2.15 (br d, *J* = 12.7 Hz, 1H), 2.05 (br d, *J* = 12.2 Hz, 1H), 1.97 - 1.86 (m, 2H), 1.85 - 1.75 (m, 6H), 1.73 - 1.52 (m, 6H), 1.37 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 110.5, 85.4, 72.3, 59.2, 41.2, 37.6, 37.2, 36.7, 36.0, 35.6, 33.5, 26.7, 26.5, 25.5. LC-MS (ESI) calculated for $C_{15}H_{24}O_3$ *m/z* $[M+Na]^+$ = 275.16, found 275.11.



Scheme S4. Synthesis of FIPC-1. All compounds are diastereomeric mixtures with the side chain located at either C-5 or C-7 of the adamantane ring as indicated in the FIPC-1 structure above.

methyl 5'-(2-((*tert*-butyldiphenylsilyl)oxy)ethyl)-5'-methylspiro [adamantane-2,3'-[1,2,4]trioxolane]-5-carboxylate (S8). A round bottom flask was charged with oxime **S7** (0.294 g, 1.24 mmol, 1.50 equiv.), ketone **S2** (0.270 g, 0.83 mmol, 1.00 equiv.), and carbon tetrachloride (20 mL). The reaction mixture was cooled in an ice bath while ozone was bubbled through the reaction mixture for 1 h. Additional oxime **S7** (0.294 g, 1.24 mmol, 1.50 equiv.) was added in 2 portions and ozone was bubbled through the reaction for another 3 h. The reaction flask was then purged with O_2 for 10 min followed by argon for 15 min. The reaction mixture was then concentrated and the residue purified on a 25 g column of silica gel with gradient elution (1-8% EtOAc in hexanes) to obtain the desired product as colorless oil (60 mg, 13%).

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 7.70- 7.67 (m, 4H), 7.46 - 7.37 (m, 6H), 3.86 - 3.76 (m, 2H), 3.67 (s, 3H, major diastereomer), 3.65 (s, 3H, minor diastereomer), 2.24 - 2.02 (m, 5H), 1.99 - 1.75 (m, 8H), 1.72 - 1.56 (m, 2H), 1.48 (s, 3H), 1.06 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 177.2, 177.1 (minor diastereomer), 135.5, 133.6, 133.6 (minor), 129.6 (minor), 129.6, 127.6, 110.6, 110.5 (minor), 109.2, 109.2 (minor), 59.9 (minor), 59.9, 51.7 (minor), 51.7, 40.5, 40.5 (minor), 39.8, 39.4, 38.1 (minor), 38.1, 36.3, 36.1, 36.1, 36.1, 35.9, 35.8, 35.6, 35.5, 33.5, 33.6, 26.8, 26.5, 26.1, 23.6 (minor), 23.6, 19.1 (minor), 19.1. LC-MS (ESI) calculated for $C_{32}H_{42}O_6Si$ *m/z* $[M+Na]^+$ = 573.26, found 573.27.

5'-(2-((*tert*-butyldiphenylsilyl)oxy)ethyl)-5'-methylspiro[adamantane-2,3'-[1,2,4]trioxolane]-5-carboxylic acid (S9). To a round bottom flask charged with **S8** (60 mg, 0.1 mmol, 1.0 equiv.), THF (1mL) and MeOH (1 mL), was added 1 M LiOH aqueous solution (0.325 mL, 0.325 mmol, 3.0 equiv.). The reaction mixture was stirred at 45 °C for 6 h and concentrated by rotary evaporation. The crude residue was taken into EtOAc (5 mL), cooled in an ice bath, and adjust the pH to 2 by adding 0.1 M HCl dropwise. The aqueous layer was extracted with EtOAc (5 mL x 2), and then the organic layers were combined, dried over anhydrous Na_2SO_4, filtered, and concentrated. The crude residue was purified on a 12 g column of silica gel with gradient elution (5–50% EtOAc in hexanes) to obtain the desired product as a colorless oil (31 mg, 53%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 7.77 - 7.59 (m, 4H), 7.46 – 7.36 (m, 6H), 3.86 – 3.76 (m, 2H), 2.21 (br d, *J* = 12.4 Hz, 1H), 2.10 - 1.77 (m, 9 H), 1.72 - 1.54 (m, 2H), 1.49 (s, 3H, minor diastereomer), 1.48 (s, 3H, major diastereomer), 1.31 - 1.23 (m, 2H), 1.06 (s, 9H), 0.96 - 0.77 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 182.5, 135.6, 133.6, 129.6, 127.7, 110.4, 109.3, 59.9, 40.5, 39.6, 39.2, 37.8, 36.1, 35.8, 35.5, 33.5, 33.5, 29.7, 26.8, 26.4, 26.0, 23.6, 19.1. LC-MS (ESI) calculated for $C_{31}H_{40}O_6Si$ *m/z* $[M+Na]^+$ = 559.25, found 559.28.

5'-(2-((*tert*-butyldiphenylsilyl)oxy)ethyl)-5'-methyl-*N*-(prop-2-yn-1-yl)spiro[adamantane-2,3'-[1,2,4]trioxolane]-5-carboxamide (S10). A round bottom flask was charged with **S9** (31 mg, 0.056 mmol, 1.0 equiv.), 4-dimethylaminopyridine (4

mg, 0.033 mmol, 0.5 equiv.), HATU (26 mg, 0.068 mmol, 1.2 equiv.), DMF (1.5 mL), and N,N-diisopropylethylamine (0.030 ml, 0.170 mmol, 3.0 equiv.). The reaction mixture was stirred for 10 min, followed by addition of propargylamine (0.004 mL, 0.067 mmol, 1.2 equiv.). The resulting reaction mixture was stirred at RT for 16 h and then diluted with EtOAc (5 mL), and washed with water (10 mL). The aqueous layer was extracted twice with EtOAc (5 mL x2) and the combined organic layers were washed with brine (5 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated. The crude product was purified on a 4 g column of silica gel with gradient elution (5–30% EtOAc in hexanes) to afford the product as colorless oil (28 mg, 87%).

^{1}H NMR (400 MHz, CDCl3, 294 K) δ (ppm) = 7.78-7.64 (m, 4H), 7.49 - 7.35 (m, 6H), 5.67 (br s, 1H), 4.15 - 3.93 (m, 2H), 3.86 – 3.72 (m, 2H), 2.25 (t, *J* = 4.0 Hz, 1H, major diastereomer), 2.23 (t, *J* = 4.0 Hz, 1H, minor diastereomer), 2.14 - 1.54 (m, 14H), 1.49 (s, 3H, minor), 1.48 (s, 3H, major), 1.26 (s, 1H), 1.05 (s, 9H); ^{13}C NMR (100 MHz, CDCl3, 295 K) δ (ppm) = 176.3, 135.6, 133.6, 129.6, 127.7, 110.4, 109.3, 79.7, 71.7, 59.8, 40.5, 39.6, 38.2, 36.5, 36.3, 36.0, 35.9, 35.7, 35.5, 33.5, 33.5, 29.3, 26.8, 26.2, 23.6, 23.6, 19.1. LC-MS (ESI) calculated for $C_{34}H_{43}NO_5Si$ *m/z* $[M+H]^+$ = 574.30, found 574.25.

5'-(2-hydroxyethyl)-5'-methyl-*N*-(prop-2-yn-1-yl)spiro[adamantane-2,3'-[1,2,4]trioxolane]-5-carboxamide (FIPC-1). A round bottom flask charged with **S10** (28 mg, 0.049 mmol, 1.0 equiv.) and THF (1 mL) was cooled at in an ice bath. To the reaction mixture was added 1 M tetrabutyl ammonium fluoride solution in THF (0.244 ml, 0.244 mmol, 5.0 equiv.) dropwise and the reaction mixture slowly warmed to RT and stirred for 16 h. The reaction mixture was then diluted with EtOAc (5 mL), and washed with water (5 mL x2). The aqueous layers were combined and back-extracted with EtOAc (5 mL). The organic layers were combined, washed with brine (5 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated. The crude residue was purified on a 4 g column of silica gel with gradient elution (20–75% EtOAc in hexanes) to obtain the product as colorless oil (16 mg, 98%).

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 5.78 (br s, 1H), 4.12 - 3.95 (m, 2H), 3.88 - 3.73 (m, 2H), 2.26 – 2.22 (m, 1H), 2.18 - 1.98 (m, 9H), 1.96 - 1.80 (m, 5H), 1.77 - 1.63 (m, 2H), 1.51 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) = 176.1, 176.1 (minor

diastereomer), 110.9, 110.3 (minor), 110.2, 79.6, 79.6 (minor), 71.7, 58.6 (minor), 58.6, 39.8, 39.7, 39.6 (minor), 39.3 (minor), 38.2, 38.1 (minor), 36.6, 36.5, 36.5 (minor), 36.4 (minor), 36.1, 35.9 (minor), 35.7, 35.6 (minor), 33.7 (minor), 33.6, 33.5, 29.3, 26.5 (minor), 26.2, 23.6. LC-MS (ESI) calculated for $C_{18}H_{25}NO_5$ *m/z* $[M+H]^+$ =336.18, found 336.03.

References

(1) Wang, X.; Dong, Y.; Wittlin, S.; Creek, D.; Chollet, J.; Charman, S. A.; Tomas, J. S.; Scheurer, C.; Snyder, C.; Vennerstrom, J. L. Spiro- and Dispiro-1,2-Dioxolanes: Contribution of Iron(II)-Mediated One-Electron vs Two-Electron Reduction to the Activity of Antimalarial Peroxides. *J. Med. Chem.* **2007**, *50*, 5840–5847.

(2) Szklarczyk, D.; Gable, A. L.; Lyon, D.; Junge, A.; Wyder, S.; Huerta-Cepas, J.; Simonovic, M.; Doncheva, N. T.; Morris, J. H.; Bork, P.; Jensen, L. J.; Mering, C. von. STRING V11: Protein-Protein Association Networks with Increased Coverage, Supporting Functional Discovery in Genome-Wide Experimental Datasets. *Nucleic Acids Res.* **2019**, *47*, D607–D613.

(3) Doncheva, N. T.; Morris, J. H.; Gorodkin, J.; Jensen, L. J. Cytoscape Stringapp: Network Analysis and Visualization of Proteomics Data. *J. Proteome Res.* **2019**, *18*, 623–632.

(4) Hangauer, M. J.; Viswanathan, V. S.; Ryan, M. J.; Bole, D.; Eaton, J. K.; Matov, A.; Galeas, J.; Dhruv, H. D.; Berens, M. E.; Schreiber, S. L.; McCormick, F.; McManus, M. T. Drug-Tolerant Persister Cancer Cells Are Vulnerable to GPX4 Inhibition. *Nature* **2017**, *551*, 247–250.

(5) Magtanong, L.; Ko, P.-J.; To, M.; Cao, J. Y.; Forcina, G. C.; Tarangelo, A.; Ward, C. C.; Cho, K.; Patti, G. J.; Nomura, D. K.; Olzmann, J. A.; Dixon, S. J. Exogenous Monounsaturated Fatty Acids Promote a Ferroptosis-Resistant Cell State. *Cell Chem. Biol.* **2019**, *26*, 420–432.e9.

(6) Forcina, G. C.; Conlon, M.; Wells, A.; Cao, J. Y.; Dixon, S. J. Systematic Quantification of Population Cell Death Kinetics in Mammalian Cells. *Cell Syst.* **2017**, *4*, 600–610.e6.

(7) Dixon, S. J.; Patel, D. N.; Welsch, M.; Skouta, R.; Lee, E. D.; Hayano, M.; Thomas, A. G.; Gleason, C. E.; Tatonetti, N. P.; Slusher, B. S.; Stockwell, B. R. Pharmacological Inhibition of Cystine-Glutamate Exchange Induces Endoplasmic Reticulum Stress and Ferroptosis. *Elife* **2014**, *3*, e02523.

(8) Guan, S.; Price, J. C.; Prusiner, S. B.; Ghaemmaghami, S.; Burlingame, A. L. A Data Processing Pipeline for Mammalian Proteome Dynamics Studies Using Stable Isotope Metabolic Labeling. *Mol. Cell Proteomics* **2011**, *10*, M111.010728.

(9) Clauser, K. R.; Baker, P.; Burlingame, A. L. Role of Accurate Mass Measurement (+/- 10 Ppm) in Protein Identification Strategies Employing MS or MS/MS and Database Searching. *Anal. Chem.* **1999**, *71*, 2871–2882.

(10) Yang, W. S.; SriRamaratnam, R.; Welsch, M. E.; Shimada, K.; Skouta, R.; Viswanathan, V. S.; Cheah, J. H.; Clemons, P. A.; Shamji, A. F.; Clish, C. B.; Brown, L. M.; Girotti, A. W.; Cornish, V. W.; Schreiber, S. L.; Stockwell, B. R. Regulation of Ferroptotic Cancer Cell Death by GPX4. *Cell* **2014**, *156*, 317–331.

(11) Cajka, T.; Fiehn, O. LC–MS-BasedLipidomics and Automated Identification of Lipids Using the LipidBlastIn-Silico MS/MS Library. In *Lipidomics:Methods and Protocols*; Bhattacharya, S. K., Ed.; Springer: New York, 2017; pp. 149–170.

(12) Cajka, T.; Smilowitz, J. T.; Fiehn, O. Validating Quantitative Untargeted Lipidomics Across Nine Liquid Chromatography-High-Resolution Mass Spectrometry Platforms. *Anal. Chem.* **2017**, *89*, 12360–12368.

(13) Tsugawa, H.; Cajka, T.; Kind, T.; Ma, Y.; Higgins, B.; Ikeda, K.; Kanazawa, M.; VanderGheynst, J.; Fiehn, O.; Arita, M. MS-DIAL: Data-Independent MS/MS Deconvolution for Comprehensive Metabolome Analysis. *Nat. Methods* **2015**, *12*, 523–526.
(14) Gaschler, M. M.; Andia, A. A.; Liu, H.; Csuka, J. M.; Hurlocker, B.; Vaiana, C. A.; Heindel, D. W.; Zuckerman, D. S.; Bos, P. H.; Reznik, E.; Ye, L. F.; Tyurina, Y. Y.; Lin, A. J.; Shchepinov, M. S.; Chan, A. Y.; Peguero-Pereira, E.; Fomich, M. A.; Daniels, J. D.; Bekish, A. V.; Shmanai, V. V.; Kagan, V. E.; Mahal, L. K.; Woerpel, K. A.; Stockwell, B. R. FINO2 Initiates Ferroptosis through GPX4 Inactivation and Iron Oxidation. *Nat. Chem. Biol.* **2018**, *14*, 507–515.
(15) Blank, B.; Gonciarz, R.; Talukder, P.; Gut, J.; Legac, J.; Rosenthal, P. J.; Renslo, A. R. Antimalarial Trioxolanes with Superior Drug-Like Properties and In Vivo Efficacy. *ACS Infect. Dis.* **2020**.
(16) Spangler, B.; Fontaine, S. D.; Shi, Y.; Sambucetti, L.; Mattis, A. N.; Hann, B.; Wells, J. A.; Renslo, A. R. A Novel Tumor-Activated Prodrug Strategy Targeting Ferrous Iron Is Effective in Multiple Preclinical Cancer Models. *J. Med. Chem.* **2016**, *59*, 11161–11170.
(17) Muir, R. K.; Zhao, N.; Wei, J.; Wang, Y.-H.; Moroz, A.; Huang, Y.; Chen, Y.-C.; Sriram, R.; Kurhanewicz, J.; Ruggero, D.; Renslo, A. R.; Evans, M. J. Measuring Dynamic Changes in the Labile Iron Pool in Vivo with a Reactivity-Based Probe for Positron Emission Tomography. *ACS Cent. Sci.* **2019**, *5*, 727–736.